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Reduction in the Number of Varicella-Zoster Virus-Specific T-Cells in Immunocompromised Children with Varicella

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Varicella zoster virus (VZV) causes a life-threatening infection in immunocompromised hosts. The immune response to VZV of healthy subjects has been rigorously assessed, but little is known about that of immunocompromised individuals. This study aimed to clarify the primary response to VZV infection in immunocompromised children. This prospective study enrolled six immunocompromised children (median age, 33 months; range, 20-62) receiving steroids or immunosuppressants, and 10 immunocompetent children (median age, 32 months; range, 15-81) with varicella. The immunocompromised children were three patients with acute lymphoblastic leukemia, two recipients with liver transplantation and one patient with juvenile idiopathic arthritis. Interferon-*γ*-producing $CD69^+$T-cells produced by VZV stimulation (VZV-specific T-cells) were studied during the acute or convalescent phase. To further address the direct effect of immunosuppressants, we analyzed the number of VZV-specific T-cells after stimulating peripheral blood mononuclear cells obtained from healthy adults with live-attenuated VZV with or without prednisolone, cyclosporine-A, or tacrolimus. The circulating numbers of lymphocytes in the convalescent stage but not acute stage were lower in immunocompromised children compared with immunocompetent children. In the acute stage, immunocompromised patients showed lower VZV-specific $CD8^+$T-cell counts than immunocompetent subjects. In contrast, in the convalescent phase, immunocompromised patients had lower VZV-specific $CD4^+$T-cell counts than immunocompetent hosts. The *in vitro* culture of activated lymphocytes with prednisolone or immunosuppressants significantly decreased the proportion of VZV-specific $CD4^+$T-cells. In conclusion, the decreased numbers of VZV-specific $CD8^+$T-cells during the acute phase and VZV-specific $CD4^+$T-cells during the convalescent phase of disease may account for severe varicella in immunocompromised children.

Keywords: cellular immunity; chickenpox; immunosuppressive agents; interferon-gamma; varicella-zoster virus
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Introduction

Varicella zoster virus (VZV) causes varicella (*chickenpox*) in susceptible persons as the primary infection and then occasionally induces zoster (*shingles*) on reactivation of the virus. Cellular immunity has a central role in the elimination of VZV and subsequent control of the latent infection (Vossen et al. 2005; Vermont et al. 2014). Varicella usually occurs in childhood, and zoster develops in the setting of reduced VZV-specific cellular immunity with age (Arvin et al. 1996; Weinberg et al. 2017). In healthy children, varicella is a benign and self-limiting illness, whereas VZV often leads to a life-threatening infection in immunocompromised patients (Matsuzaki et al. 2008). The diagnosis of primary infection of this virus in immunocompromised patients is difficult because the vis-

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ceral involvement may precede cutaneous manifestations or develop in the absence of any skin lesions (Rowland et al. 1995; Matsuzaki et al. 2008). Dermatomal localization of zoster is the most common form of the reactivation of VZV in healthy subjects, while the dissemination and/or organ involvement may arise from the primary infection as well as the reactivation in immunocompromised patients (Ishizaki et al. 2003; Umezawa et al. 2014). A previous study indicated that the high incidence of zoster in patients with systemic lupus erythematosus was associated with reduced VZV-specific $CD4^+$T-cell responses, which were recognized as the number or proportion of interferon-γ-producing $CD69^+$ $CD4^+$T-cells after the stimulation of VZV antigen (Park et al. 2004). However, the critical factors for the development of disseminated VZV infections remain unclear. There is little information about the differences in the initial immune response and the establishment of VZV-specific immunity between immunocompromised and immunocompetent children with varicella.

To identify the mechanism underlying progressive VZV disease, we studied the dynamics of VZV-specific T-cells in immunocompromised children during the course of varicella. The direct effect of immunosuppressive agents on the VZV-specific T-cells was also investigated *in vitro* using the culture of peripheral blood mononuclear cells (PBMCs) obtained from healthy adults.

Materials and Methods

Subjects

This prospective study enrolled six immunocompromised and 10 immunocompetent children who received a diagnosis of varicella due to primary infection between January 1, 2011, and December 31, 2014 at the Department of Pediatrics in Kyushu University Hospital, or the National Hospital Organization Fukuoka Higashi Medical Center. No patients had a history of varicella. Only one immunocompromised child had a history of one-time VZV vaccination. The diagnosis of varicella was based on the clinical findings including typical vesicular eruption at each erythematous base.

The immunocompromised children (median age, 33 months; range, 20-62) were three patients with acute lymphoblastic leukemia (ALL), two recipients with liver transplantation and one patient with juvenile idiopathic arthritis (JIA). There was no evidence of primary immunodeficiency diseases or human immunodeficiency virus infection. Two of the ALL patients were on maintenance chemotherapy. At the onset of varicella, one of them received dexamethasone, vincristine, methotrexate and 6-mercaptopurine, and another patient with infant ALL received dexamethasone, vincristine, methotrexate, 6-mercaptopurine, etoposide, cyclophosphamide, high-dose cytarabine and L-asparaginase. The remaining one with relapsed ALL was on re-induction chemotherapy consisting of dexamethasone, vincristine, cyclophosphamide, cytarabine, 6-mercaptopurine, pirarubicin and L-asparaginase. The two liver transplant recipients were in stable conditions posttransplant on tacrolimus therapy. The JIA patient had controlled disease activity on tocilizumab therapy. Previously healthy children who suffered from varicella served as the immunocompetent controls (median age, 32 months; range, 15-81). Informed consent was obtained from all patients' parents.

Collection of samples

Heparinized peripheral blood samples were collected from patients at the diagnosis of varicella (acute phase) and 2-8 weeks after the onset (convalescent phase). Whole-blood samples were partly used for the subsequent flow-cytometry to examine for proportion of lymphocyte subsets. PBMCs were separated from the remaining samples via density-gradient centrifugation using LSM (Cappel-ICN Immunobiologicals, Costa Mesa, CA, USA) to perform the analysis of VZV-specific T-cells. Leukocyte counts were routinely measured in the hospital laboratories. All examinations were started within 24 hours after the sample collection.

Analyses of lymphocyte subsets

A three- or four-color flow cytometric analysis was performed for at least 20,000 lymphocytes by using an EPICS XL (Beckman Coulter) as described previously (Honda et al. 2000). Phycoerythrin (PE)-conjugated anti-CD3 antibody (BD Biosciences, Mountain View, CA, USA), fluorescein isothiocyanate (FITC)-conjugated anti-CD19 antibody (Beckman Coulter, Miami, FL, USA) and phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-CD56 antibody (Beckman Coulter) were used to determine the proportions of T-cells, B-cells and natural killer (NK) cells, respectively, among gated lymphocytes. FITC-conjugated anti-CD4, PE-conjugated anti-CD3 and PC5-conjugated anti-CD8 antibodies (Beckman Coulter) were used to determine helper and cytotoxic T-cells.

Measurement of VZV-specific cells

PBMCs separated from heparinized whole-blood samples were stimulated for 16 hours at 37℃ under 5% CO_2 with a live attenuated Oka strain of VZV [> 2,000 plaque-forming units (pfu)/mL, BIKEN, Osaka, Japan] in the culture medium [RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) plus 10% fetal calf serum (MP Biomedicals, Santa Ana, CA, USA)] containing gentamycin (MSD, Tokyo, Japan), according to the established method for the determination of VZV antigen-specific cells (Vossen et al. 2004). The PBMCs without stimulation were also cultured under the same condition for 16 hours in the same culture medium (negative control). After the stimulation, the PBMCs were further cultured for 4 hours with 10 μg/mL brefeldin. PBMCs without the stimulation were also exposed to 25 ng/mL phorbol 12-myristate acetate (PMA; Sigma Chemical, St Louis, MO, USA) plus 1 μg/mL ionomycin (Sigma Chemical) in the culture medium (positive control).

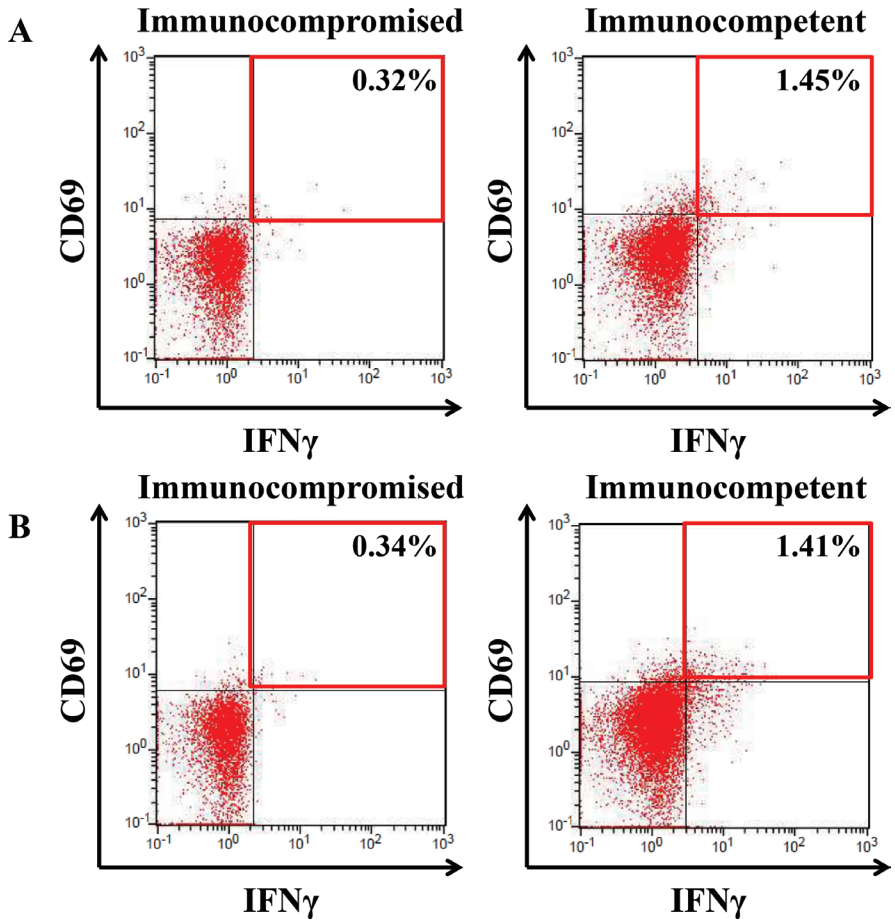

Fig. 1. Representative flow cytometry dot plots.
Blood leukocytes were defined by gating on $CD4^+$ (A) and $CD8^+$ T-cells (B). The proportions of $IFN\gamma^+CD69^+$ cells in $CD4^+$ (A) and $CD8^+$ T-cells (B) were compared between immunocompromised and immunocompetent children.

After the incubation, each sample (0.1 mL) was stained with phycoerythrin-Texas Red (ECD)-conjugated anti-CD3 and PC5-conjugated anti-CD4 or CD8 antibodies. The lysis of erythrocytes and membrane permeabilization were performed as previously described (Hoshina et al. 2012). After these steps, PBMCs were incubated for 30 min with FITC-conjugated anti-interferon (IFN)γ and PE-conjugated CD69 antibodies (Beckton Dickinson). The cells were then washed and resuspended in phosphate-buffered saline and analyzed by flow cytometry (EPICS XL, Beckman Coulter, Fig. 1). The proportion of VZV-specific cells was defined as a value that was obtained by subtracting the number of IFNγ-producing CD69-positive T-cells without VZV stimulation from the cells with the stimulation (Fig. 2).

In vitro stimulation of PBMCs with immunosuppressive agents

PBMCs were collected from 10 healthy adults positive for VZV IgG antibody after informed consent was obtained. PBMCs were cultured after the stimulation of a live attenuated Oka strain of VZV (> 2,000 pfu/mL) with or without the addition of prednisolone (100 μg/mL), cyclosporine-A (CsA) (100 ng/mL) or tacrolimus (10 ng/mL) (Wako Pure Chemical Industries, Osaka, Japan) for 16 hours at 37°C in a 5% CO_2 incubator, according to the established method (Pearl et al. 2005). The subsequent analysis of IFNγ-producing CD69-positive T-cells (VZV-specific T-cells) was performed according to the aforementioned protocol.

Statistical analyses

Wilcoxon's rank-sum test was used to compare the group means between two groups. Fisher's exact test was applied to determine the distribution between two categorical variables. Wilcoxon's signed-rank test was used to evaluate the variation of VZV-specific T-cells by *in vitro* stimulation with immunosuppressive agents. *P* values of less than 0.05 were considered to indicate statistical significance. The statistical software program JMP® version 9.0 (SAS Institute, Cary, NC, USA) was used for the analysis.

Ethical approval

The present study was approved by the Institutional Review Board of Kyushu University.

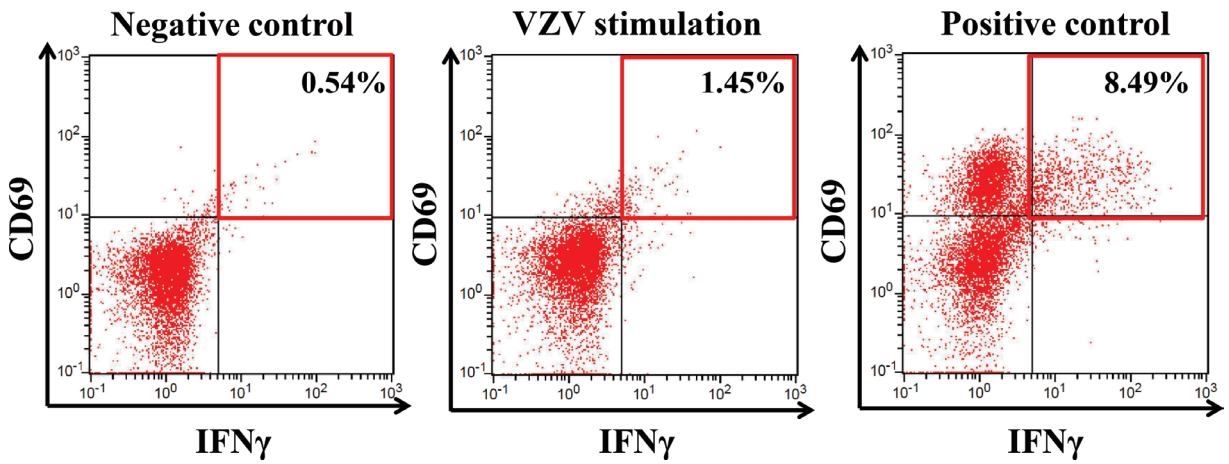

Fig. 2. The flow cytometry dot plots in an immunocompetent patient.
Negative control indicates the cells without stimulation, varicella zoster virus (VZV) stimulation indicates the cells that were stimulated with a live attenuated Oka strain of VZV, and positive control indicates the cells with PMA and ionomycin. The proportion of VZV-specific cells was defined as a value that was obtained by subtracting the number of IFNγ-producing CD69-positive T-cells without VZV stimulation from the cells with the stimulation.

Results

Clinical characteristics of varicella in the eligible patients

The demographic and clinical characteristics in patients with varicella by each group are shown in Table 1. There were no significant differences in the demographics and clinical expressions or in the VZV-specific IgM titers or plasma viral loads between the two groups. All patients received acyclovir or valacyclovir after the diagnosis of varicella, which was continued until the crust formation of all vesicles. Most immunocompromised patients were treated with intravenous acyclovir, whereas many immunocompetent patients were treated with oral acyclovir or valacyclovir, except for three patients with the poor oral intake and the difficulty in taking an oral drug. The duration of antiviral therapy in immunocompromised patients (median, 8 days; range, 5-15) was longer than that in immunocompetent patients (median, 5 days; range, 2-6, $P = 0.0045$). No patients died during the study period.

Analysis of VZV-specific cells in the acute stage of varicella

During the acute phase, there was no marked difference in the absolute numbers of peripheral leukocytes, lymphocytes, T-cells, $CD4^+$T-cells, $CD8^+$T-cells, B-cells or NK cells between immunocompromised and immunocompetent patients (Table 2). The numbers and proportions of $CD8^+CD69^+$ IFNγ-producing T-cells after the stimulation with VZV vaccine (VZV-specific $CD8^+$T-cells) were each significantly lower in immunocompromised patients than in immunocompetent patients (number $P = 0.012$, Table 2; proportion $P = 0.0022$, Fig. 3B). In contrast, there was no significant difference in the values of $CD4^+CD69^+$ IFNγ-producing T-cells after the stimulation with VZV vaccine (VZV-specific $CD4^+$T-cells) between the two groups (Table 2, Fig. 3A).

The analysis of VZV-specific cells in the convalescent stage of varicella

During the convalescent phase, the absolute numbers of lymphocytes ($P = 0.030$) and $CD4^+$T-cells ($P = 0.040$) but not total leukocytes or other lymphocyte subsets were lower in immunocompromised patients than in immunocompetent patients (Table 3). In contrast to the acute stage, the numbers and proportions of $CD4^+CD69^+$ IFNγ-producing T-cells after the stimulation with VZV vaccine were each lower in immunocompromised patients than in immunocompetent patients (number $P = 0.017$, Table 3; proportion $P = 0.0039$, Fig. 3C). In contrast, there was no significant difference in those of $CD8^+CD69^+$ IFNγ-producing T-cells after the stimulation with VZV vaccine between the two groups (Table 3, Fig. 3D).

Effects of immunosuppressive agents on VZV-specific T-cells of healthy donor

VZV specific T-cells (IFNγ-producing $CD69^+$T-cells after the stimulation with VZV) were then assessed using PBMCs obtained from 10 healthy adult donors positive for anti-VZV IgG antibody subjects. After the attenuated VZV stimulation with or without prednisolone, CsA or tacrolimus, an intracellular flow cytometric analysis was performed. The proportion of $CD4^+CD69^+$ IFNγ-producing T-cells was lower in cultures with these agents than in those without them (each P value 0.016, Fig. 4). However, there was no marked difference in the proportion of $CD8^+CD69^+$ IFNγ-producing T-cells between the cultures with and without these agents. The $CD69^+$ IFNγ-producing T-cells after stimulation with phorbol 12-myristate acetate plus ionomycin as a positive control was augmented in all PBMC samples (data not shown).

Discussion

The present study demonstrated for the first time the

Table 1. Demographics and clinical profiles of the patients with the primary infection of VZV.

	Immunocompromised n = 6	Immunocompetent n = 10	*P* value
Age, months	33 [20-62]	32 [15-81]	1.00
Gender, % male	2 (33.3)	5 (50.0)	0.63
History of varicella, n (%)	0 (0)	0 (0)	N.S.
VZV vaccination, n (%)	1 (16.7)	0 (0)	0.38
Fever, n (%)	3 (50.0)	6 (60.0)	1.00
preceding skin eruption, n (%)	0 (0)	2 (33.3)	0.50
Skin eruption, n (%)	6 (100)	10 (100)	1.00
preceding fever, n (%)	3 (50.0)	4 (40.0)	1.00
hemorrhagic/giant bullous lesions, n (%)	0 (0)	0 (0)	N.S.
spread from body to extremities/from ext. to body, n	6 / 0	10 / 0	N.S.
Organ dysfunction, n (%)	3 (50.0)	1 (10.0)	0.12
liver dysfunction, n (%)	1 (16.7)	1 (10.0)	1.00
ileus, n (%)	0 (0)	0 (0)	N.S.
VZV-IgM levels†	0.27 [0.14-6.67]	1.38 [0.38-5.94]	0.09
VZV DNA in plasma, copies/mL†	600 [200-2,000]	200 [0-900]	0.37
Days of illness at the beginning of antiviral therapy	2 [2-3]	2 [1-3]	0.10
Duration of antiviral therapy‡	8 [5-15]	5 [2-6]	0.0045
Proportion of intravenous antiviral therapy, n (%)	5 (83.3)	3 (30.0)	0.12
Fatal cases, n (%)	0 (0)	0 (0)	N.S.

Data represent the median and ranges, and the values in parenthesis mean the proportion. The data were analyzed using the Wilcoxon rank-sum test and the Fisher's exact test. *P* values < 0.05 were considered statistically significant.
VZV, varicella zoster virus; N.S., not significant.
†The antibody titer or viral load was measured for limited number of samples (IgM 5 *vs.* 7, DNA 3 *vs.* 5) at diagnosis. Under detection limit values (< 200 copies/mL) were represented as zero.
‡Antiviral therapy was continued until the crust formation of all vesicles. The dose of acyclovir was 20 mg/kg orally four times daily or 5 mg/kg intravenously three times daily. The dose of valacyclovir was 25 mg/kg orally three times daily.

Table 2. Absolute number of lymphocytes and IFNγ producing cells during the acute phase of VZV infection.

	Immunocompromised n = 6	Immunocompetent n = 10	*P* value
Leukocyte count, /μl	7,265 [2,950-13,360]	7,070 [3,100-21,380]	0.59
Lymphocyte count, /μl	1,376 [122-6,613]	2,479 [1,005-5,467]	0.45
T-cell† count, /μl	946 [12-4,473]	1,794 [570-3,642]	0.45
$CD4^+$ T-cell count, /μl	589 [10-2617]	1,236 [275-2,453]	0.23
$CD8^+$ T-cell count, /μl	316 [2-1,570]	684 [189-1,295]	0.45
B-cell† count, /μl	270 [1-1,292]	401 [330-1,432]	0.45
NK cell† count, /μl	65 [2-368]	77 [13-292]	0.83
IFNγ-producing cells‡			
$CD4^+CD69^+$ cell count, /μl	0 [0-6]	4 [0-21]	0.23
$CD8^+CD69^+$ cell count, /μl	0 [0-1]	1 [0-27]	0.012

Data represent the median and ranges. The data were analyzed using the Wilcoxon's rank-sum test.
P values < 0.05 were considered statistically significant.
VZV, varicella zoster virus; IFNγ, interferon-gamma; NK cell, natural killer cell.
†T-cells, B-cells and NK cells were defined as peripheral blood mononuclear cells exclusively positive for CD3, CD19 and CD56, respectively.
‡These cells were counted after the stimulation with a live attenuated VZV vaccine.

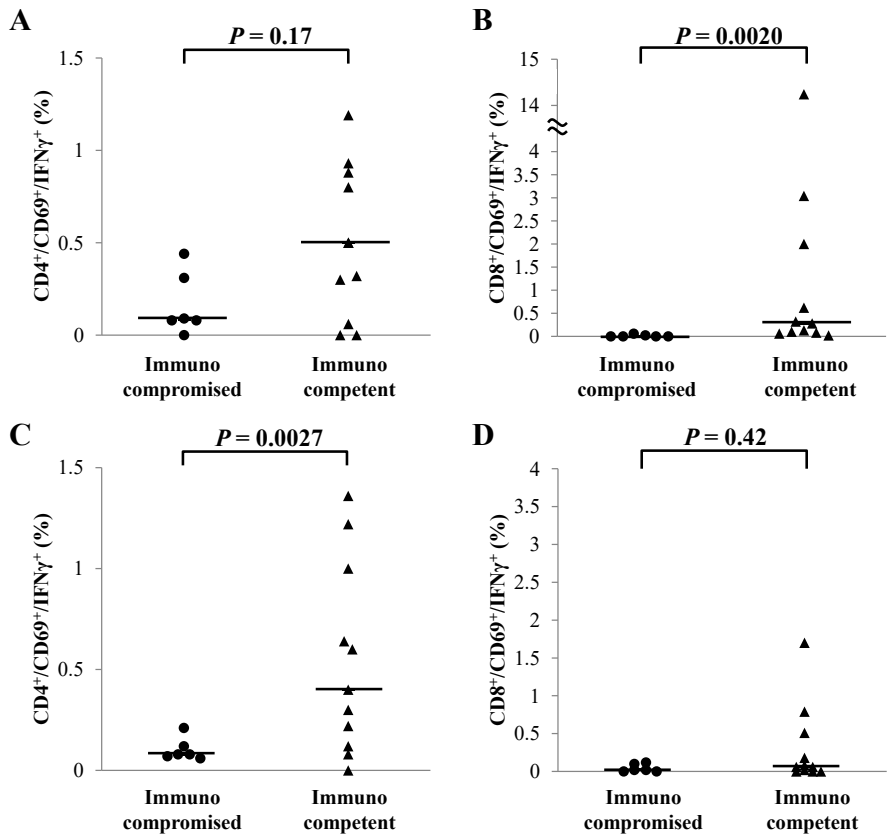

Fig. 3. The proportions of VZV-specific T-cells in immunocompromised and immunocompetent children with varicella. The proportions of IFNγ^+CD69$^+$ cells in CD4$^+$ (A) and CD8$^+$ (B) T-cells in the acute phase of varicella were compared between the 2 groups. The proportions of IFNγ^+CD69$^+$ cells in CD4$^+$ (C) and CD8$^+$ (D) T-cells in the convalescent phase of varicella were compared between the 2 groups. "CD4$^+$/CD69$^+$/IFNγ^+" and "CD8$^+$/CD69$^+$/IFNγ^+" at the Y axis mean the proportions of VZV-specific T cells among CD4$^+$ and CD8$^+$ T cells, respectively. Horizontal bars indicate the median values. Circular and triangular plots indicate the values of immunocompromised and immunocompetent children, respectively. The data were analyzed using the Wilcoxon rank-sum test. *P* values < 0.05 were considered statistically significant.

Table 3. Absolute number of lymphocytes and IFNγ producing cells during the convalescent phase of VZV infection.

	Immunocompromised n = 6	Immunocompetent n = 10	*P* value
Leukocyte count, /μl	4,820 [1,950-15,770]	10,585 [5,610-14,300]	0.19
Lymphocyte count, /μl	2,065 [546-8,547]	4,378 [2,893-7,688]	0.030
T-cell† count, /μl	1,170 [501-5,427]	2,843 [1,515-5,273]	0.051
CD4$^+$ T-cell count, /μl	427 [345-3,137]	1,578 [736-3,021]	0.040
CD8$^+$ T-cell count, /μl	432 [131-1,465]	1,010 [457-1,941]	0.28
B-cell† count, /μl	303 [5-1,803]	1,070 [399-1,874]	0.065
NK cell† count, /μl	150 [10-574]	173 [31-520]	0.74
IFNγ-producing cells‡			
CD4$^+$CD69$^+$ cell count, /μl	0 [0-3]	6 [1-26]	0.017
CD8$^+$CD69$^+$ cell count, /μl	0 [0-1]	1 [0-33]	0.27

Data represent the median and ranges. The data were analyzed using the Wilcoxon's rank-sum test. *P* values < 0.05 were considered statistically significant.
IFNγ, interferon gamma; VZV, varicella zoster virus; NK cell, natural killer cell.
†T-cells, B-cells and NK cells were defined as peripheral blood mononuclear cells exclusively positive for CD3, CD19 and CD56, respectively.
‡These cells were counted after the stimulation with a live attenuated VZV vaccine.

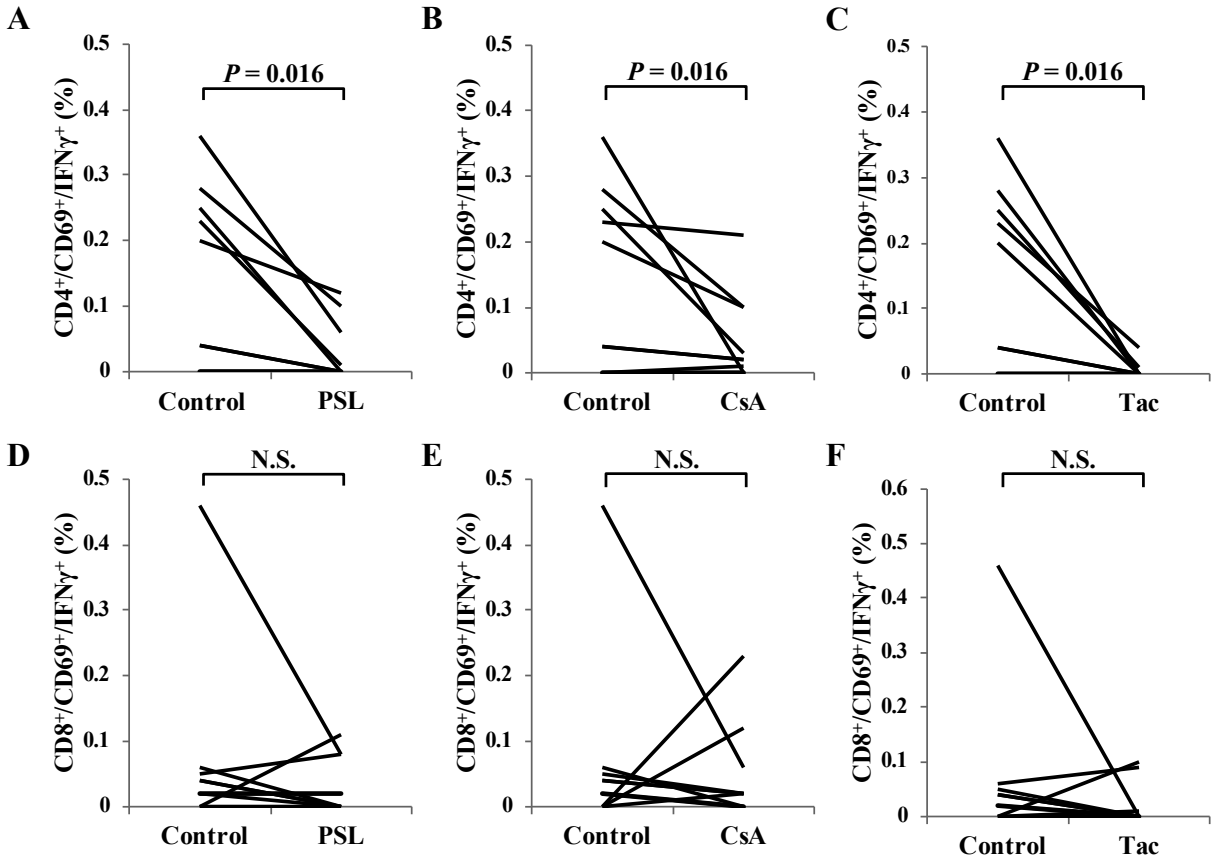

Fig. 4. The proportions of interferon-γ-producing CD69-positive T-cells stimulated with attenuated VZV and an immunosuppressive agent.
(A)-(C) The proportions of interferon-γ-producing $CD4^+CD69^+$ T-cells under the stimulation with an attenuated VZV and prednisolone (A), cyclosporine-A (B) or tacrolimus (C). (D)-(F) The proportions of interferon-γ-producing $CD8^+CD69^+$ T-cells under the stimulation with an attenuated VZV and prednisolone (D), cyclosporine-A (E) or tacrolimus (F). Control indicates the analysis which is performed using peripheral blood mononuclear cells (PBMCs) under the stimulation with only an attenuated VZV. Others indicate the analysis which is performed using PBMCs under the stimulation with an attenuated VZV and prednisolone, cyclosporine-A or tacrolimus. "$CD4^+/CD69^+/IFN\gamma^+$" and "$CD8^+/CD69^+/IFN\gamma^+$" at the Y axis mean the proportions of VZV-specific T cells among $CD4^+$ and $CD8^+$ T cells, respectively. The data were analyzed using the Wilcoxon signed-rank test. *P* values < 0.05 were considered statistically significant. PSL, prednisolone; CsA, cyclosporine-A; Tac, tacrolimus; IFNγ, interferon gamma; N.S., not significant.

decreased number of VZV-specific T-cells in secondary immunocompromised children after primary infection of the virus. In the acute stage of varicella, these patients showed a decreased number of VZV-specific $CD8^+$T-cells compared with immunocompetent children. In contrast, in the convalescent phase, they had lower numbers of VZV-specific $CD4^+$T-cells than immunocompetent subjects. VZV-specific T-cells play a central role in the elimination of the virus and also control the reactivation (Arvin et al. 1996; Vossen et al. 2005; Vermont et al. 2014; Weinberg et al. 2017). The decreased number of VZV-specific $CD8^+$ and $CD4^+$ T-cells may derange the phase-specific control of virus replication in immunocompromised children.

The immune responses to VZV at the onset of zoster have been analyzed in many reports (Steain et al. 2014; Schub et al. 2015), whereas few studies have investigated the responses in the acute phase of varicella. The present study demonstrated the appearance of more effector T cell subpopulation in immunocompetent children than in immunocompromised children with primary infection of VZV. $CD4^+$T-cells have a great impact on the control of VZV infection (Haberthur et al. 2011). Cytotoxic $CD8^+$T-cells also play a critical role in restraining the virus replication (Steain et al. 2014). Irrespective of primary infection or reactivation, VZV infection leads to severe disease in immunocompromised patients (Ishizaki et al. 2003; Matsuzaki et al. 2008). The circulating viral load practically reflects the severity of VZV infection (Ishizaki et al. 2003). However, in the present study, the copy number of VZV DNA in immunocompromised patients was not significantly higher than that seen in immunocompetent patients at the onset of varicella. This supports the policy of making an early diagnosis and performing timely treatment via antiviral therapy for immunocompromised patients with an ambiguous onset of varicella. In this line, prolonged acyclovir therapy might result in the favorable outcomes of all

immunocompromised patients, although the optimum duration of treatment has not yet been established.

The major concern is the mechanisms of progressive VZV infection in immunocompromised patients. Vossen et al. (2005) indicated that the number of VZV-specific $CD8^+$T-cells in patients with severe primary VZV infection was lower than that in patients with non-severe varicella, suggesting that $CD8^+$T-cells effectively suppress the replication of virus after primary infection. Because the starting time of antiviral therapy did not differ markedly between the two groups, the low number of VZV-specific $CD8^+$T-cells in immunocompromised patients might corroborate the delayed recovery from varicella. However, $CD4^+$T-cells are associated with the decreased number of the replication of VZV during the convalescent phase as well as that during acute phase (Schub et al. 2015). $CD4^+$T-cells effectively prevent the reactivation of VZV (Park et al. 2004). The reduction of VZV-specific $CD4^+$T-cells is a major risk factor of the development of zoster (Duncan and Hambleton 2015). Immunocompromised hosts, such as patients with SLE and hematopoietic stem cell transplantation recipients, have a low number of VZV-specific $CD4^+$T-cells (Offidani et al. 2001; Park et al. 2004; Rondaan et al. 2014). The number of $CD4^+$T-cells declines with age even in healthy adults, which leads to an increased susceptibility to the development of zoster (Kawai et al. 2014; Weinberg et al. 2017). In the present study, in immunocompromised children, the number of VZV-specific $CD4^+$T-cells remained low even in the convalescent phase of primary VZV infection when the number of these effector T-cells is expected to increase (Vossen et al. 2005; Weinberg et al. 2017). In addition, the proportion of VZV-specific effector cells in $CD4^+$T-cells was significantly decreased in the culture of adult PBMCs with prednisolone, CsA or tacrolimus. Although our experiment did not provide any direct evidence, these findings suggest that the prolonged administration of prednisolone or immunosuppressive drugs may directly suppress the induction of memory T-cells during the primary infection of VZV, thereby impairing the long-term maintenance of the memory function. As an alternative possibility, the apoptosis of T-cells may be caused by some of these drugs (Guchelaar et al. 1998; Herold et al. 2006; Choi et al. 2008).

Immunocompromised patients occasionally suffer from a visceral involvement without any cutaneous lesion after primary infection of VZV (Rowland et al. 1995; Matsuzaki et al. 2008). The skin-sparing effect in dissemination has not been fully understood. The present study did not verify the difference in the immune response to VZV infection between typical varicella and visceral varicella, as all eligible immunocompromised patients had typical skin lesions. The numbers of VZV-specific $CD8^+$ and $CD4^+$ T-cells in some immunocompromised patients were negligible after the onset of varicella, but their clinical findings were the similar to those of immunocompetent children. The antiviral therapy was started early, because all eligible immunocompromised patients had skin eruptions during the early phase of VZV infection. Therefore, there might be no significant difference in the severity of symptoms related to the VZV infection between immunocompromised and immunocompetent patients. In addition, cellular immunity of the eligible immunocompromised patients might have been maintained relatively, compared to those with severe VZV infection, although we could not compare the number of T-cells between these two groups. Moreover, it is speculated that innate immunity is also associated with the appearance of the primary symptoms. We tried to analyze VZV-specific natural killer cells and $\gamma\delta$T-cells which act as innate immune cells, but the absolute numbers of these cells were too small to compare between immunocompromised and immunocompetent patients. Further studies are needed to analyze circulating VZV-specific T-cells as well as innate immune cells in patients with visceral varicella.

The present study has some limitations. First, not all immunocompromised children develop varicella due to primary infection of VZV, as varicella can be caused as both primary infection and reactivation of the virus in immunocompromised hosts. The serum antibody titers of VZV-IgM do not always indicate primary infection, as VZV reactivation also induces IgM antibodies (Kangro et al. 1988). To demonstrate serologic primary VZV infection, we measured VZV-IgG antibody titers using serum samples obtained before the development of varicella. Although the titers were negative in two of the six patients, those of the remaining four patients were positive (data not shown). These four patients had received intravenous immunoglobulin for the control of each underlying disease; namely, VZV-IgG titers in these patients were not helpful to determine the primary infection or reactivation of VZV. Primary VZV infection unexceptionally expresses the clinical manifestation of viremia (Arvin 1996). Therefore, each eligible patient in this study was determined to have primary infection of VZV based on comprehensive information. Second, in immunocompromised patients, the immune status of each patient was heterogeneous because the type of immunosuppressants and therapy varied among the patients. Third, a live VZV vaccine with an unconfirmed viral load was used to stimulate PBMCs. However, all products included > 2,000 pfu/mL of the virus, and PBMCs were appropriately stimulated in previous studies using this live vaccine (Landmeier et al. 2007; Yu et al. 2011). Fourth, analyses of the subpopulations of $CD4^+$ and $CD8^+$T-cells (e.g., memory T-cells) were not performed in the present study. Finally, the study population was relatively small, which may have affected the accuracy of the statistical analysis.

In conclusion, VZV-specific $CD8^+$T-cells and $CD4^+$T-cells were not driven in immunocompromised children with varicella, which may explain their delayed recovery and lethal infection. The VZV vaccination is useful for preventing varicella and zoster in immunocompromised hosts; however, the efficacy and adverse events depend on the

magnitude of their immune response to the virus (Banovic et al. 2011). The enhancement of herd immunity by the active vaccination of healthy individuals should therefore be promoted for the safe and effective control of the primary infection of VZV in immunocompromised patients.

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Conflict of Interest

The authors declare no conflict of interest.

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